# EFFECT OF FERMENTATION TIME ON BUTANOL AND ETHANOL PRODUCTION FROM PALM MILL OIL EFFLUENT BY *CLOSTRIDIUM ACETOBUTYLICUM*

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# ABSTRACT

Biofuels production through fermentation from renewable waste has been put more to focus in mid 20$^{th}$ century till now. Main focus of the research is to determine optimum fermentation time to obtain maximum concentration of butanol and ethanol via ABE fermentation. The ABE fermentation is a two phase chemical production which is from acidogenesis into solventogenesis. The fermentation is caused by clostridia bacteria and in this case of study is *Clostridium acetobutylicum* pattered NCIMB 13357. The main substrate for nutrient in this study is Palm Oil Mill Effluent (POME) which freshly taken from Lepar Hilir, Pahang. Through the research, POME was found to contain majorly of pentose and carbohydrate substance such as galactose, glucose, fructose, sucrose and lactose. The research parameter is fermentation time at 72, 80, 48 and 60 hours. The experiments start from medium preparation, cultivation of bacteria, inoculation, fermentation and lastly analysis of product. All standard solution is made in order to standardize the reading of concentration of product and substance analyzed. The condition for bacteria environment is set to anaerobic and optimum pH at 5.8, temperature at 37 °C and agitation speed is set at 200 rpm along ABE fermentation. Final result shown that at fermentation time of 40 hours, the maximum value of 0.07 g/L of butanol was produced, while for ethanol is 83.499 g/L at 20 hour fermentation time. POME is encouragingly valid for use as fermentation media as it contain many utilizable substrates for *Clostridium acetobutylicum*.

# ABSTRAK

Penghasilan biofuel melalui fermentasi sisa buangan telah menjadi fokus pada pertengahan abad ke-21 sehingga kini. Fokus kajian ini adalah untuk menentukan masa fermentasi yang mana kepekatan maksimum butanol dan etanol menerusi fermentasi ABE. Fermentasi ABE adalah produksi kimia dua fasa yang mana dari fasa asidogenasis kepada solventogenesis. Proses ini dirangsangkan oleh bacteria clostridia yang mana dalam kajian ini adalah *Clostridia acetobutylicum* berpaten NCIMP 13357. Substrat utama sebagai nutrisi dalam kajian ini adalah sisa kelapa sawit hancuran (POME) yang diambil segar dari Lepar Hilir, Pahang. Menerusi kajian, didapati bahawa POME mengandungi pentos dan rangkain karbohidrat seperti galaktos, glukos, fruktos, sukros dan laktos. Parameter kajian adalah masa fermentasi iaitu pada 72, 80, 48 dan 60 jam. Eksperimen dimulakan dengan penyediaan media, mengkultur bacteria, inokulum, proses fermentasi dan akhirnya analisis produk. Semua larutan standad dibuat bertujuan menyelaraskan bacaan kepekatan produk dan substrat yang dianalisis. Persekitaran bakteria untuk fermentasi adalah anaerobik dengan pH optimum pada bacaan 5.8, suhu pada 37 °C, dan kelajuan goncangan pada 200 rpm sepanjang fermentasi ABE .Hasil kajian menunjukkan pada masa fermentasi 40 jam, maksimum sebanyak 0.07 g/L butanol dihasilkan, manakala untuk etanol adalah sebanyak 83.499 g/L pada 20 jam masa fermentasi. Didapati bahawa POME adalah sangat bersesuaian dan amat digalakkan sebagai media fermentasi kerana ia mengandungi pelbagai substrat yang boleh di gunakan oleh *C. acetobutylicum.*

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| | | |
|---|---|---|
| ºC | - | Degree Celsius |
| % | - | Percentage |
| ABE | - | Acetone-butanol-ethanol |
| DNS | - | DiNitroSalicyclic acids |
| GC-FID | - | Gas Chromatography Flame Ionized Detector |
| g/L | - | gram per liter |
| g | - | Gram |
| HPLC | - | High Performance Liquid Chromatography |
| hr | - | hour |
| L | - | Liter |
| mL | - | milliliter |
| POME | - | Palm Mill Oil Effluent |
| rpm | - | Revolution Per Minute |
| w/v | - | Weight per volume |
| w/v % | - | Weight per volume in percentage |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Background of Research

The conversion of plant biomass into solvents for fuel and chemical industry is regarded as old technology founded in the early 20$^{th}$ century (Zverlov *et al*., 2006). Fermentation of sugar into ethanol is in fact the oldest biotechnology in beer and wine production and regarded as the largest biotechnology process until now (Berezeina *et al.,* 2006). Biological production of solvent such as acetone, butanol and ethanol by fermentation in industrial scale has start in the early part of this century (Ranad *et al.*,2000). An early attempt L. Pasteur and others, the fermentation of starch to the solvents acetone, butanol and ethanol was developed by C. Weizman in 1912 at Manchester University into an industrial process (Velokodvorskaya *et al.,* 2006). The strain isolated and used was then called *clostridium acetobutylicum* and the first production plant was run for acetone large scale production from starch. The plant was erected in Canada and USA during World War I and during that time butanol was regarded as unnecessary by-product (Zverlov *et al.,* 2006). After war, butanol need was increased and the fermentation industry changed the substrate to molasses and the search for more applicable strain and clostridia were in highly demand (Berezeina *et al.,* 2006).

The commercial acetone-butanol fermentation process was established by the Commercial Solvent Corporation (CSC), USA which predominant until 1930's when the widespread availability of cheap molasses from the sugar industry provided a strong incentive to switch substrate (Shaheen *et al.,* 2000). Considerable effort was invested in attempting to utilize the existing starch-fermenting for use was never showing great result (Hastings *et al.,* 1971). It was then until mid 1950s, *C. acetobutylicum* was used in the profitable acetone-butanol-ethanol (ABE) fermentation in respond to the increasing demands for butanol and ethanol as biofuel an acetone as industrial chemical solvent.

*Clostridium acetobutylicum* has a long history of fermentation industry due to its biphasic fermentation of sugars to produce acetic and butyric acids in acidogenesis phase and then converted into solvent (acetone, butanol and ethanol) during solventogenesis (Xue *et al.,* 2000: Jones and Woods, 1989). Isolated by Chaim Weizmann in 1912 till 1914 at Manchester University, strains with high solvent yield are then named as *Clostridium acetobutylicum* (Velikodvorskaya *et al.,* 2006). *C. acetobutylicum* has the ability to utilize a variety of starchy substances as fermentation media (Jones and Woods, 1986). Early 20$^{th}$ century, starch and molasses are the main substrate for fermentation (Zverlov *et al.,* 2006), due to increase demand for solvent such as butanol, acetone and ethanol for many application (Kwang *et al.,* 2008), the cost for substrate has a dramatic influence on the economic viability of fermentation for ABE fermentation production (Nasratun et al., 2007). An interest has been taken in considering waste as substrate such as palm oil mill effluent (POME) due to the typical characteristic of the POME itself (Takriff *et al.,* 2007).

Malaysia was the largest producer of palm oil after Indonesia (Nasratun *et al.,* 2007). Its production generates various wastes chief among which is POME from production of crude palm oil involving extraction process (Sahaid *et al.,* 2003). The large amount production along the years has solved the availability of POME as substrate for ABE fermentation and can be considered as sustainable resources. POME

is classify as highly concentrated industrial waste water with BOD up to 40,000 mg/L thus may result in serious pollution if waste management is at critical point stand-up (Pang *et al.,* 2003). From the result of a research by Khaw, 1999, the report showed that *C. acetobutylicum* produced a total of ABE up to 0.94 g/L when grown in POME as the fermentation medium. Not only is POME contain mixture of carbohydrates including starch, hemicelluloses, sucrose and other carbohydrates which can be utilize by saccharolytic clostridia such as *C. acetobutylicum* (Kwon *et al*., 1989; Mohtar *et al.,* 2003), such utilization can result large increase profitability of palm oil industry, solving environmental problem and reducing the cost for economical solvent production via ABE fermentation (Yoshino *et al.,* 2003).

## 1.2 Problem Statement

Currently, ABE fermentation is a value-added fermentation process as it attractive for several economics and environmental factors (Formanek *et al.,* 1997). Environmental and global energy problems have resulted in increase effort towards producing biofuels such as butanol and ethanol from renewable resources (Youngsoon *et al.,* 2009) such as agriculture residue or waste. Agriculture waste is produced in a great amount and sometimes has effect on environment. For example is POME as it contains highly concentration of BOD of 40,000 mg/L (Pang *et al.,* 2003). On an average a palm oil mill, for each tonne of fresh fruit bunch (FFB) processed, 1 tonne of liquid or waste water with biochemical oxygen demand (BOD) 37.5kg, chemical oxygen demand (COD) 75kg, suspended solids (SS) 27kg and oil and grease 8kg was generated alongside (Zinatizadeh *et al.,* 2007). In an aspect of biofuels economy, although biofuels such as biodiesel and bioethanol represent secure, renewable and environmentally safe alternative compared to fossil fuel, their economic viability in term of costing has become a major concern (Gonzalez *et al.,* 2007). As a result, application of waste-to-wealth concept as main ideas for more profitable and safer strategy is in run. Usage of POME as fermentation media has

become recent interest research on Malaysia and other palm oil country as it may help add-on economy profit, reducing waste management issues and costing and also contribute on green house effect reduction. Other than environmental and economy viability, health aspect also been considered as the main focus. Butanol from petro-derivatives carries carcinogen effect (Thaddeus *et al.,* 2007) thus fermentation derived butanol is preferred specifically usage on food and flavor industry (Ezeji *et al.,* 2007). Some strain (Clostridia strain family) pattern later after 1940s allowed fermentation times to be as short as 30 hour fermentation time (Shaheen *et al.,* 2000). With some strain, long fermentation time such as up to 72 hour resulted in lower solvent productivity (Matt *et al*., 2000). These two fact has up rise the issues of effective fermentation time in obtaining the target solvent namely butanol and ethanol.

## 1.3 Objectives of Researches

The objective of the research is to study the effect of fermentation time on butanol and ethanol production by *C. acetobutylicum*.

## 1.4 Scope of Research

There are three scope of the research. The scopes are as follow:

1. To complete the composition analysis of selected batch fresh POME by using HPLC analysis.

2. To analyze the glucose consumption throughout the fermentation using the UV-Vis and glucose calibration curve by concentration estimation.

3. To study the effect of fermentation time at 72, 80, 48 and 60 hours of ABE fermentation by *C. acetobutylicum*.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Fermentation

Fermentation is describes a form of energy-yielding microbial metabolism in which carbohydrate acts as the electron acceptor (Adams, 1990). In a meaning is a process involving ethanol production by yeast or organic acids by certain bacteria such as clostridia family bacteria (Sahlin, 1999). Generally fermentation is applied in food industry to produce fermented food containing lactic acids for solving dehydration health problem (Peter, 1999) by children mainly. Fermentation can be aerobic or anaerobic. For anaerobic fermentation, ABE fermentation is the most in research at 21st century.

### 2.1.1 Anaerobic Fermentation

Anaerobic fermentation is a biological processes where organic matter is metabolized in and environment free of dissolved oxygen (Kumar *et al.*, 2008). Certain microorganism can produce adenosine triphosphate (ATP) by utilizing

metabolic pathways that do not require the participation of molecular oxygen. The process is named as anaerobic glycolysis or pathways of fermentation and can be simplify as anaerobic fermentation. One type of anaerobic condition derived bacteria is clostridia family strain as it can utilize sugars into organic acids and further fermentation can produce solvents (Blaschek, 2005). Anaerobic fermentation has recently focused more on waste water treatment with the recovery of useful byproducts and renewable biofuels by using anaerobic biotechnology (Samir, 2008).

### 2.1.2 ABE Fermentation

The acetone-butanol-ethanol (ABE) fermentation of *C. acetobutylicum* was a classical method to produce the commercially important solvents such as acetone, butanol and ethanol and operated successfully at an industrial scale in many countries during the first half of $20^{th}$ century (Yang *et al.*, 2007) process has received considerable attention in recent years as a method to produce commodity chemicals, such as butanol and acetone, from biomass. It was carried out industrially throughout the United State during the half of last century, but was discontinued in the early 1960s due to unfavorable economic conditions brought about the competition with the petrochemical industry (Ezeji *et al.*, 2000). The ABE fermentation is the most widely studied among the anaerobic fermentation processes and is a model for complex primary metabolism fermentations (Blascheck *et al.*, 2000). Below are the pathways of how does the fermentation proceed throughout the fermentation (Thaddeus *et al.*, 2007). The ABE fermentation is round-up mainly on biphasic concept and the change between. The phase shifts is from acidogenesis to solventogenesis and happen upon certain specific time interval throughout the fermentation. On early stage, it started with acidogenesis where the main product is butyric acid, acetic acid and small amount of other organic acid (Ahmed *et al.*, 1988). After a few hour, the fermentation process proceed to the next step where the phase are then change into solventogenesis,

where solvent such as butanol, ethanol, acetone and phenol are produce. At this process, almost all of the initial acids produced were to be converted by the Clostridia Acetobutylicum into solvent at certain ratio depending on the condition and many other factors such as fermentation time, temperature, agitation speed and concentration of substrate.

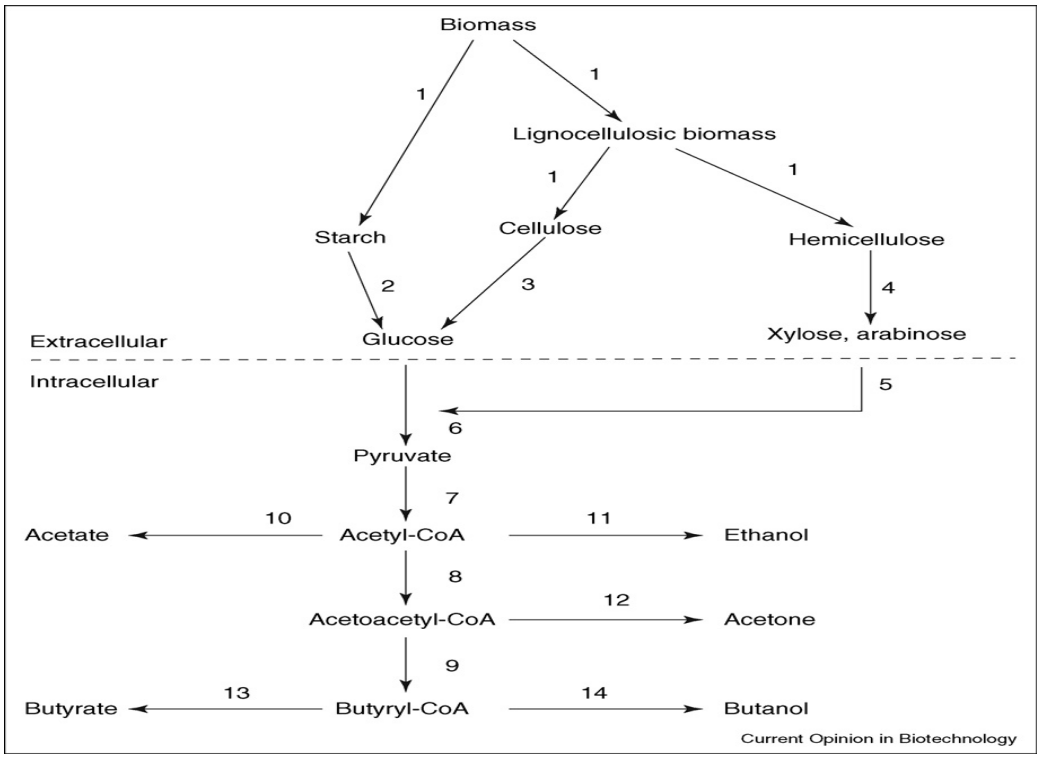


**Figure 2.1** : Simplified metabolism of biomass by solventogenic clostridia. 1, Pretreatment of corn and lignocellulose; 2, starch hydrolysis (a-amylase,b-amylase, pullulanase, glucoamylase, a-glucosidase); 3, cellulose hydrolysis (cellulases, b-glucosidase); 4, hemicellulose hydrolysis; 5, xylose/arabinose uptake and subsequent breakdown via the transketolase-transaldolase sequence producing fructose 6-phosphate and glyceraldehydes 3-phosphate with subsequent metabolism by the Embden-Meyerhof-Parnas (EMP) pathway; 6, glucose uptake by the phosphotransferase system (PTS) and conversion to pyruvate by the EMP pathway; 7, pyruvate-ferrodoxin oxidoreductase; 8, thiolase; 9, 3- hydroxybutyryl-CoA dehydrogenase, crotonase and butyryl-CoA dehydrogenase; 10, phosphate acetyltransferase and acetate kinase; 11, acetaldehyde dehydrogenase and ethanol dehydrogenase; 12, acetoacetyl-CoA:acetate/butyrate:CoA transferase and acetoacetate decarboxylase; 13, phosphate butyltransferase and butyrate kinase; 14, butyraldehyde dehydrogenase and butanol dehydrogenase (Thaddeus *et al.*, 2007).

## 2.2 Butanol and Ethanol

Butanol and ethanol are the main solvent targeted in ABE fermentation as these two are the most potential biofuels. These two alcohol is the major solvents produced during fermentation at high ratio compare to other side product and these has become the targeted beneficial in finding the alternative fuels.

### 2.2.1 Butanol

Butanol is widely used as an industrial chemical. Butanol also exhibit a range of physical properties, including high energy content, water immiscibility low vapor pressure, and octane-enhancing power, which provide it with potential as a liquid fuel (Zhen *et al.*, 2008). The development of fermentation processes based on the solventogenic clostridia offers the prospect of butanol production from agriculture feedstock as an alternative to the petrochemical route (Blaschek *et al.*, 2008)

### 2.2.2 Ethanol

Ethanol (ethyl alcohol, grain alcohol) is a clear, colorless liquid with a characteristic, agreeable odor. It has low freezing characteristic which made it useful as the fluid in thermometers for temperature below -40 $^{o}$C, the freezing point of mercury, and for other low-temperature purposes, such as for antifreeze in automobile radiators. Ethanol has been made since ancient times by fermentation of sugar (glucose, fructose etc). All beverage ethanol and more than half of industrial ethanol is still made by this process. The ethanol produced by fermentation ranges in concentration from percent up to 14 percent (Shakhashiri *et al.*,2001).

## 2.3 Clostridia Acetobutylicum

The main clostridia used for the experiment is *Clostridium acetobutylicum* coding pattern NCIMB 13357. Below are the table of the strain characteristic and identification.

**Table 2.1** : Characteristic and pattern for *Clostridium acetobutylicum* NCIMB 13357 (Keis *et al* 2001). The incubation and also the fermentation temperature as shown are at $37^0C$; Gram strain is gram positive.

| **Clostridium acetobutylicum** | |
|---|---|
| Accession Number: | 13357 |
| Species Name: | Clostridium acetobutylicum |
| Other Collection: | ATCC824 DSM792 NRRLB527 VKMB1787 |
| Date of Accession: | 19/05/1995 00:00:00 |
| Depositor Name: | H. Hippe |
| Type Strain: | Yes |
| Isolated by: | E.R.Weyer (Granulobacter pectinovorum) |
| **Growth** | |
| Growth Medium: | Clostridium acetobutylicum medium |
| Incubation Temp: | 37°C |
| Gas Regime: | anaerobic |
| **Colony Morphology** | |
| Gram Strain: | gram positive |
| **Preservation Information** | |
| Method: | lyophilized |

## 2.4 Palm Oil Mill Effluent (POME) Potential as Fermentation Medium.

POME has a great potential as alternative fermentation media due to its rich content of sugar and carbohydrate which can be utilize by C.Acetobutylicum for ABE fermentation. POME consist of various suspended components including cell walls, organelles and short fibers, a spectrum of carbohydrates ranging from hemicelluloses to simple sugar, a range of nitrogenous compound from proteins to amino-acids and free organic acids (Ugoji ,1997). The simple sugars contained within POME are fructose, glucose, galactose, sucrose and lactose. Due to the flexibility of *C. acetobutylicum* in using variety of starchy substances for media of fermentation (Jones and Wood, 1986), in these past 20 year, continuous experiment and research has been conducted in trying variety of starchy substance such as glucose, molasses, starch and corn. However, the cost of substrate has been the major consideration in determining the economy viability of ABE fermentation through anaerobic fermentation (Ezeji *et al.,* 2007). This means that availability of cheaper, abundant and readily available sources of substrates such as POME should be taken as alternative media for fermentation (Nasratun *et al.,* 2007) rather than other expansive material such as corn. Also, this can solve environment and water pollution issues since POME is greatly produced and can polluted the environment. This way also, economy viability can be enhanced via ABE fermentation process. Shown here is the table showing the typical characteristic of POME (Ahmad *et al.* 2006).

**Table 2.2** : Characteristics of POME (palm oil mill effluent) (Ahmad *et al.* 2006). (As shown below, the BOD and COD reach a highly concentrated value and pose threat into environment (Pang *et al.*, 2003). Not to mention the total solid and suspended solid value and the difficulties of disposing the solid waste issues).

| Parameter | Concentration (mg/L) | Element | Concentration (mg/L) |
|---|---|---|---|
| Oil & Grease | 4000-6000 | Phosphorus | 180 |
| BOD | 20,000-25,000 | Potassium | 2270 |
| COD | 40,000-50,000 | Calcium | 439 |
| Total Solids | 40,500 | Boron | 7.6 |
| Suspended Solid | 18,000 | Iron | 46.5 |
| Total volatile solids | 34,000 | Manganese | 2.0 |
| Ammoniccals nitrogen | 35 | Copper | 0.89 |
| Total | 750 | Magnesium<br><br>zinc | 615<br><br>2.3 |

# CHAPTER 3

# METHODOLOGY

## 3.1 Material

In this research, the main materials used in conducting the experiment is RCM powder, for inoculation and fermentation media (RCM powder) and agar (RCM agar powder); 5M NaOH for titration on POME media in maintaining pH at 5.8 (Kalil *et al.,* 2003); DNS reagent stored at 4°C (glucose presence analysis); distilled water (for media, sterilization in autoclave, sanitization); Toluene solution (LLE process); sugars solution for standard calibration curve (glucose, galactose, fructose, sucrose, and lactose); Nitrogen gas for anaerobic condition and media sparging; ethanol 70% for sterilization and sanitization; Acetonitrile solution for HPLC mobile phase; Hexane solution for GC-FID; $Na_2SO_4$ for LLE water absorption; POME media for fermentation media in studies. As for apparatus, test tubes; modified schott bottle; retort stand and holder; metal clipper; syringe 10 and 20mL, syringe filter 0.5 and 0.2mm; syringe needles; 60 vials; 40 GC-FID and HPLC vials; water bath; silica tube and lastly the Nitrogen gas supply.

## 3.2 Equipments

Equipment used majorly in this research is HPLC, GC-FID, autoclave, centrifuge, anaerobic chamber, incubator shaker and UV-Vis spectrophotometer.

### 3.2.1 HPLC

High performance liquid chromatography (HPLC) is a powerful tool in analysis for multi component in a solution. It is basically a highly improved form of column chromatography. Rather than solvent/solution being allowed to drip through a column under gravity, it is forced through under high pressure of up o 400 atmospheres, making it much faster. It also allows a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows better separation of the component of the mixture. The column is filled with tiny silica particles, and the solvent is non-polar such as hexane. A typical column has an internal diameter of 4.6 mm or less and length of 150 to 250 mm. The time taken for particular compound to travel through the column to the detector is known as its retention time. The time measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound (Jim, 2007). In this study, the HPLC was set to use capillary column Supelcosil LC-NH2. The flow rate used is 1ml/minute and the retention time around 15 minutes.

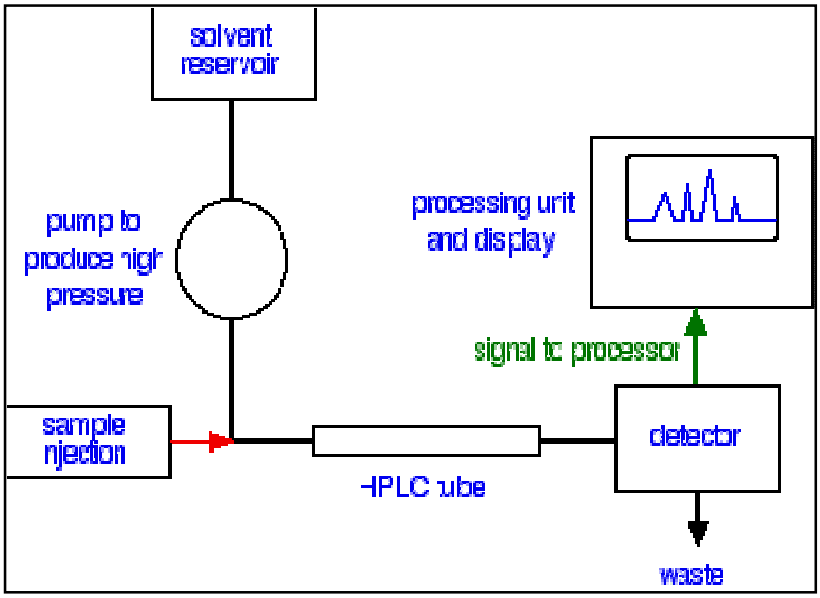


**Figure 3.1** : Flow scheme for HPLC, the sample was injected and pass through HPLC tube (column) and after separation it pass through in separate retention time and detected by detector. After that, the value (area unit) was displayed and the waste was collected.

### 3.2.2 GC-FID

Gas chromatography flame ionization detector (GC-FID) is a non-selective detectoused in conjunction with gas chromatography. The FID works by directing the gas phase output from the column into a hydrogen flame. A voltage of 100 to 200 volt is applied between the flame and an electrode located away from the flame. The increased current due to electron emitted by burning carbon particles is then measured. The FID detect all carbon containing compound .The detector also has an extremely wide linear dynamic range that extends over, at least five orders of magnitude with a response index between 0.98 to 1.02.